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(54) Title: STIMULATION OF ANGIOGENESIS AND PROMOTION OF ENDOTHELIALISATION

(57) Abstract

A method of stimulating angiogenesis or endothelialisation in a mammal, comprising the step of administering to that mammal an anti-inflammatory compound. The anti-inflammatory compound preferably has a directly-acting angiogenic effect; preferred compounds include salicyclic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid. An anti-inflammatory compound may be admninistered together with a second stimulator of angiogenesis. Compositions and articles are also claimed.

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# STIMULATION OF ANGIOGENESIS AND PROMOTION OF ENDOTHELIALISATION

This invention relates to the control of angiogenesis, and methods and compositions therefor.

According to one aspect of the present invention there is provided a method for stimulating angiogenesis in a mammal, characterized by the use of an anti-inflammatory agent.

Knowledge of factors controlling proliferation of the endothelium is essential for understanding the molecular and cellular basis of the normal process of capillary

formation and of pathological process such as abnormal retinal vasoproliferation leading to blindness, and tumor-induced angiogenesis.

Full identification of the references cited hereinafter will be found at the end of this specification.

By studying the migratory and proliferative responses of cultured endothelial cells it should be possible to identify those substances that might be involved in regulation of neovascularisation. A number of polypeptide growth factors has been shown to enhance vascular endothelial cell proliferation in vitro. These include 3T3-cell derived growth factor (McAuslan et al., 1980), tumor-derived growth factor (Klagsbrun et al., 1982) and endothelial cell growth stimulator (ECGF) (Maciag et al., 1981).

The induction of new blood vessel growth and formation of a vascular network is elicited in animals by extracts of carcinoma cells (Folkman, 1974) or of normal bovine parotid glands (Fleming, 1959). Partially purified fractions of quite low-molecular-weight substances (200-300 Dalton) from Walker carcinoma (McAuslan and Hoffman, 1979; Weiss et al 1979; Fenselau et al., 1981), bovine parotid glands, or bovine liver (McAuslan et al., 1981) have been shown to be angiogenic by ocular implant or chick chorioallantoic membrane assays. It has been shown that low concentrations of copper ions can induce neovascularisation in the anterior eye chamber or corneal pocket and also migration of endothelial cells in culture (McAuslan, 1979; McAuslan and Gole, 1980; McAuslan and Reilly, 1980).

Thus a wide variety of agents has been shown to be capable of inducing angiogenesis in various assay systems. Some of these agents appear to act via a leukocyte-mediated mechanism, since the response is blocked by pretreatment of the test animals with corticosteroids.

It is known that some of the mediators produced in response to an inflammatory stimulus are angiogenic. Because of undesirable side effects of inflammation, an ideal agent for control of angiogenesis should have a direct action, and

should itself be anti-inflammatory. A further limitation is imposed by the necessity for the agent to penetrate the target organ.

It has previously been suggested that aspirin and indomethacin could affect endothelial cells and that aspirin had some angiogenic activity; however, it was thought that this activity was mediated via leucocytes (McAuslan and Gole, 1980)

I have now found that a number of potent anti-inflammatory compounds are angiogenic as assessed by their ability to stimulate migration of capillary endothelial cells in vitro and to induce angiogenesis in vivo. I have further found that aspirin exerts a directly-acting angiogenic activity.

The mechanism of action of these compounds is unclear, but may be related to inhibition of either glycoprotein or prostaglandin synthesis. Without wishing to be bound by any postulated or hypothetical mechanism for the observed beneficial effects, it is noted that the active compounds possess an aromatic carboxylic acid group.

Classes of anti-inflammatory compounds whose structure includes an aromatic carboxylic acid group are summarized in Table 1.

TABLE 1

CLASSES OF ANTI-INFLAMMATORY COMPOUNDS WHOSE STRUCTURE INCLUDES AN AROMATIC CARBOXYLIC ACID MOIETY

| Salicylates | Benzene acetic acid derivatives | Anthranilic acid derivatives | Phenyl acetic acid derivatives | Thiazole acetic acid derivatives |
|---|---|---|---|---|
| N-acetyl salicyclic acid (aspirin) | Ibuprofen | Diclofenac (see also phenylacetic acid derivatives | Alclofenac | Fenclozic acid |
| salicyclic acid | Indoprofen | Etofenamate | Diclofenac | |
| salicylamide | Ketoprofen | Flufenamic acid | Fenclofenac | |
| Diflunisal | | Meclofenamic acid | Fenclorac | |
| Fendosal | | Mefenamic acid | Ibufenac | |
| | NB also called hydratropic acids 2-phenylpropanoic acids | | | |

According to one aspect of the present invention there is provided a method of stimulating angiogenesis in a mammal, comprising the step of administering to that animal an anti-inflammatory compound.

According to another aspect of the invention there is provided a method of stimulating endothelialisation in a mammal, comprising the step of administering to that mammal an anti-inflammatory compound.

The anti-inflammatory compound is preferably selected from the group which includes salicylic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid, and their angiogenically-active analogues and derivatives.

Preferably the anti-inflammatory compound has a directly-acting angiogenic effect.

Preferably the compound comprises an aromatic carboxylic acid group.

Most preferably the compound is administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

Combinations of two or more compounds according to the invention may optionally be used.

Combinations of one or more compounds according to the invention together with one or more other stimulators of angiogenesis may also optionally be used. Said second stimulator is suitably a modulator of collagen synthesis or of collagen fibril assembly.

Preferably the modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

More preferably, the inhibitory agent is selected from the group which includes cis-4-hyroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their angiogenically-active analogues and derivatives. Alternatively, said second stimulator of angiogenesis is epidermal growth factor or a pharmacologically active analogue, fragment or derivative thereof.

The compound according to the invention may optionally be administered in a slow-release form or in a biodegradable matrix.

We have used two principal assay systems to test compounds for their ability to stimulate or inhibit angiogenesis. The corneal pocket assay in rabbits as described by Gimbrone et al. (1974) was used according to the modification of McAuslan and Gole (1981). However, in this system it is extremely difficult to distinguish a directly acting angiogenic stimulus from one which is mediated by leukocytes (McAuslan et al., 9183). Since endothelial cell migration is a primary event in neovascularisation, and since there is a correlation between the ability of certain metal ions to induce vascularisation and their ability to cause migration of cultured cells, such migration has been suggested (McAuslan 1979) as the basis for a quantitative assay of angiogenic activity. There is comparatively little information on the correlation between this activity and neovascularising activity, and furthermore, a number of unrelated substances will induce migration of cultured endothelial cells and neovascularisation (McAuslan 1979) Proliferation of endothelial cells is thought to be a response secondary to cell migration during new vessel formation. There are reports of low-molecular-weight neovasculogenic activities that can stimulate proliferation of cultured endothelial cells. However, the proliferative responses have been marginal and the reports are not in accord as to the minimal conditions or cell type necessary.

I have found that compounds which stimulate endothelial cell migration are always angiogenic. However because of the role of inflammatory mediators in some angiogenic systems, the converse is not necessarily true. Consequently, as a further confirmation of angiogenic activity, I have used an assay system in which an annular ring of silicone containing a matrix of highly purified atelocollagen in which is embedded a 1 $mm^3$ fragment of slow-release copolymer of polyethylene-vinyl acetate impregnated with the agent to be tested is implanted

subcutaneously (S/C) into rabbits. This polymer is biocompatible and non-inflammatory, and the assay is highly sensitive.

Materials and Methods

Polymer Preparation.

Slow-release polymers of ethylene vinyl acetate (Elvax 60, trade mark of Polysciences Corp.) were prepared by the method of Langer and Folkman (1976). For ocular assays, sterile fragments of approximately 1 $mm^3$ were used and for the chorioallantois assay, approximately 2 $mm^3$.

Rabbit Subcutaneous Implant Assay

An annular ring of silicone containing a matrix of highly purified atelocollagen in which is embedded a 1 $mm^3$ fragment of slow-release copolymer of polyethylene-vinyl acetate impregnated with the agent to be tested is implanted subcutaneously into each rabbit.

Each polymer fragment is impregnated with approximately 0.5 mg of the solid agent to be tested, so that the agent diffuses out of the polymer and sets up a concentration gradient which changes with time.

Corneal Pocket Assay

The corneal pocket assay of Gimbrone et al (1974) as modified by Gole and McAuslan (1981) was used on New Zealand white rabbits of 2-3kg body weight. Opposite eyes of each animal were used as control and test, respectively. The results were documented photographically and histologically 10 days postoperation.

Endothelial Cells

Clonal lines of bovine aortal endothelial cells, whose growth and maintenance was as described by McAuslan et al (1982) were used. Similar results were obtained with either type of cell line.

A line of bovine retinal capillary endothelial cells free from mural cells was established essentially by the procedures of Buzney and Massicotte (1979).

Cell Migration Assays

The procedure for studying induced endothelial cell migration as well as the quantitation of average track lengths has been presented in detail by McAuslan and Reilly (1980).

The invention will be illustrated by reference to the following non-limiting examples.

Example 1

Anti-inflammatory agents were tested for angiogenic activity in the subcutaneous implant assay in rabbits as described above. The results are shown in Table 2. Both flufenamic acid and diclofenac showed strong activity in stimulating vascularization. Only one of twelve controls showed any activity, giving a weak response.

TABLE 2

SUBCUTANEOUS IMPLANT ASSAY

Elvax pellets contained 0.05 mg test agent per $mm^3$, i.e. equivalent to $2 \times 10^{-4}$M.

| Inducer | Total number of implants and intensity of vascularisation | | | | |
|---|---|---|---|---|---|
| | ++++ | +++ | ++ | + | - |
| Controls | 0 | 0 | 1 | 0 | 11 |
| Flufenamic acid | 3 | 6 | 2 | 1 | 0 |
| Diclofenac | 4 | 4 | 3 | 1 | 0 |

Intensity score:

++++ Large numbers of distinct blood vessels invading the gel; numerous blood vessels growing toward the silicon tube. Markedly angiogenic.

+++ Fine blood vessels invading the collagen gel; less intense than above; fine blood vessels around the silicon tube; strongly angiogenic.

| | |
|---|---|
| ++ | Slight pink around the periphery of the collagen gel due to a few fine capillaries; fine vessels around the silicon tube; weakly angiogenic. |
| + | Collagen gel unchanged; fine blood vessels growing towards the silicon tube; incipient angiogenesis. |
| - | Collagen gel unchanged; no blood vessels around the silicon tube; non-angiogenic. |

Example 2

The same agents were tested for their ability to stimulate migration of bovine capillary endothelial cells by the method of McAuslan and Reilly (1980). Results are shown in Table 3. Aspirin, flufenamic acid, and diclofenac all had strong stimulatory activity, whereas phenylbutazone was negative. It is noted that phenylbutazone does not contain an aromatic carboxylic acid group.

TABLE 3

MIGRATION ASSAYS

Bovine capillary endothelial cells

| Inducer | Conc. | % Response | Average track area ($x10^{-3}\mu m^2$) |
|---|---|---|---|
| Control | - | 0 | 15.93 |
| Flufenamic acid | $10^{-5}$M | 80 | 28.71 |
| | $10^{-6}$M | 140 | 38.23 |
| | $10^{-7}$M | 130 | 36.63 |
| Diclofenac | $10^{-5}$M | 145 | 31.31 |
| | $10^{-6}$M | 111 | 33.60 |
| | $10^{-7}$M | 76 | 28.10 |
| Aspirin | $10^{-6}$M | 63 | 26.0 |
| Phenylbutazone | $10^{-5}$M | 0 | 15.8 |
| | $10^{-6}$M | 0 | 15.0 |
| | $10^{-7}$M | 0 | 15.9 |

Example 3

Anti-inflammatory agents were tested for angiogenic activity in the corneal pocket assay as described above. Results are shown in Table 4. Both flufenamic acid and diclofenac showed strong activity. One of the six controls was positive.

TABLE 4

CORNEAL POCKET ASSAY

Elvax pellets (1 $mm^3$) contained approx. 26 ng. agent/$mm^3$ i.e. equivalent to $10^{-4}$M.

| | Left Eyes | Right Eyes |
|---|---|---|
| Controls | 1/6 | - |
| Flufenamic acid | - | 6/6 |
| Diclofenac | - | 4/6 |

Anterior Eye Chamber Assays for Aspirin:
see: McAuslan B.R. and Gole G.A. - Trans Ophthal. Soc. U.K. (1980) 100 354.

Applications of the Invention

The present invention is capable of application in a wide variety of clinical fields.

Stimulation of angiogenesis can be used to enhance the healing of burns and wounds, especially those involving large tissue defects, acceptance of skin or organ grafts, and can also be used in reconstructive and cosmetic surgery, including the use of subdermal implants, and in prosthetic surgery, particularly that involving vascular prostheses. Such stimulation may be used in any situation wherein endothelial cell migration and regeneration of endothelium are advantageous, or where an increase in blood flow is desirable, e.g., stroke, heart disease, or foetal blood insufficiency.

In particular, the method according to the invention could be used in the following situations:

a) Where development of a capillary network would be advantageous,

e.g. Surgical repair, wound healing.

b) Where stimulation of endothelialisation would be advantageous,

e.g. Synthetic or natural graft materials.

c) Where healing may be enhanced by either angiogenic or anti-inflammatory action,

e.g. Implantable prosthetic devices.

This application excludes the use of Diclofenac as an anti-inflammatory agent which might improve the performance of cardiac pacemaker electrodes.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

References cited herein are listed on the following pages.

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CLAIMS

1. A method of stimulating angiogenesis in a mammal, comprising the step of administering to that animal an anti-inflammatory compound.

2. A method of stimulating endothelialisation in a mammal, characterized by the step of administering to that mammal an anti-inflammatory compound.

3. A method according to Claim 1 or Claim 2, wherein the anti-inflammatory compound has a directly-acting angiogenic effect.

4. A method according to any one of claims 1 to 3, wherein the anti-inflammatory compound comprises an aromatic carboxylic acid group.

5. A method according to Claim 1 or Claim 2, wherein the anti-inflammatory compound is selected from the group which includes salicylic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid, and their angiogenically-active analogues and derivatives.

6. A method according to any one of Claims 1 to 5, wherein two or more anti-inflammatory compounds are used.

7. A method according to any preceding claim, wherein one or more second stimulators of angiogenesis is additionally used.

8. A method according to Claim 7, wherein the second stimulator of angiogenesis is a modulator of collagen synthesis or of collagen fibre assembly.

9. A method according to Claim 8, wherein the modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

10. A method according to Claim 9, wherein the inhibitor is selected from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their angiogenically-active analogues and derivatives.

11. A method according to Claim 7, wherein the second stimulator of angiogenesis is epidermal growth factor or a pharmacologically-active analogue, fragment or derivative thereof.

12. A method according to any preceding claim, wherein the anti-inflammatory compound is administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

13. A method according to any one of Claims 1 to 12, wherein the anti-inflammatory compound is administered in a slow-release form or in a biodegradable matrix.

14. The use of an anti-inflammatory compound for manufacture of a medicament for stimulating angiogenesis or promoting endothelialisation in a mammal.

15. The use of an anti-inflammatory compound according to Claim 14, wherein the anti-inflammatory compound has a directly-acting angiogenic effect.

16. The use of an anti-inflammatory compound according to Claim 14 or Claim 15, wherein the anti-inflammatory compound comprises an aromatic carboxyl group.

17. The use of an anti-inflammatory compound according to any one of Claims 14 to 16, wherein the anti-inflammatory compound is selected from the group which includes salicylic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid, and their angiogenically active analogues and derivatives.

18. The use of an anti-inflammatory compound according to any one of Claims 14 to 17, wherein two or more anti-inflammatory compounds are used.

19. The use of an anti-inflammatory compound according to any one of Claims 14 to 18, wherein the medicament additionally comprises one or more second stimulators of angiogenesis.

20. The use of an anti-inflammatory compound according to Claim 19 wherein the second stimulator of angiogenesis is a modulator of collagen synthesis or of collagen fibre assembly.

21. The use of an anti-inflammatory compound according to Claim 20, wherein the modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

22. The use of an anti-inflammatory compound according to Claim 21, wherein the inhibitor is selected from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their angiogenically-active analogues and derivatives.

23. The use of an anti-inflammatory compound according to Claim 19, wherein the second stimulator of angiogenesis is epidermal growth factor or a pharmacologically-active analogue, fragment or derivative thereof.

24. The use of an anti-inflammatory compound according to any one of Claims 14 to 23, wherein the medicament is adapted to be administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

25. The use of an anti-inflammatory compound according to any one of Claims 14 to 23, wherein the medicament is adapted to be administered in a slow-release form or in a biodegradable matrix.

26. A topically-applicable composition for stimulation of angiogenesis or of endothelialisation comprising an anti-inflammatory compound, together with a pharmaceutically-acceptable diluent or excipient.

27. A composition for stimulation of angiogenesis or of endothelialisation comprising an anti-inflammatory compound and a pharmaceutically-acceptable diluent or excipient, wherein the composition is adapted to release the anti-inflammatory compound over an extended period after administration.

28. A composition for stimulation of angiogenesis or of endothelialisation comprising an anti-inflammatory compound and a pharmaceutically-acceptable diluent or excipient, wherein the composition is adapted to be administered in a biodegradable matrix.

29. A composition for stimulation of angiogenesis or of endothelialisation comprising an anti-inflammatory compound and a pharmaceutically-acceptable diluent or excipient, wherein the composition is adapted to be administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

30. A composition for stimulation of angiogenesis or of endothelialisation comprising an anti-inflammatory compound and one or more second stimulators of angiogenesis, together with a pharmaceutically-acceptable diluent or excipient.

31. A composition according to Claim 30, wherein the second stimulator of angiogenesis is a modulator of collagen synthesis or of collagen fibre assembly.

32. A composition according to Claim 31, wherein the modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

33. A composition according to Claim 32, wherein the inhibitor is selected, from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their angiogenically-active analogues and derivatives.

34. A composition according to Claim 31, wherein the second stimulator of angiogenesis is epidermal growth factor or a pharmacologically-active analogue, fragment or derivative thereof.

35. A composition according to any one of Claims 30 to 34, which is adapted to be administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

36. A composition according to any one of Claims 30 to 34, which is adapted to be administered in a slow-release form or in a biodegradable matrix.

37. A subdermal implant, synthetic or natural graft material, vascular prosthesis or prosthetic device comprising one or more anti-inflammatory compounds as set out in any one of Claims 14 to 33.

38. A subdermal implant, synthetic or natural graft material, vascular prosthesis or prosthetic device comprising one or more anti-inflammatory compounds as set out in any one of Claims 14 to 33 and a second stimulator of angiogenesis as set out in any one of Claims 19 to 23.

39. Methods, compositions and articles substantially as hereinbefore described with reference to the examples.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00256

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] A61K 31/195, 31/60

## II. FIELDS SEARCHED

Minimum Documentation Searched †

| Classification System | Classification Symbols |
|---|---|
| IPC | A61K 31/195, 31/60 |
| US Cl. | 514-159, 514-165 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ‡

AU : IPC as above

## III. DOCUMENTS CONSIDERED TO BE RELEVANT §

| Category * | Citation of Document, ‖ with indication, where appropriate, of the relevant passages ‖ | Relevant to Claim No. ‖ |
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| X | AU,B, 25148/84 (560035) (WARNER-LAMBERT CO.) 25 October 1984 (25.10.84) | 1-39 |
| X | GB,A, 1032253 (FORSCHUNGS et al) 8 June 1966 (08.06.66) See page 1 lines 63-68 | 1-39 |
| X | US,A, 2515189 (BROWN et al) 18 July 1950 (18.07.50) | 1-39 |
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| X | US,A, 3928587 (SAWYER) 23 December 1975 (23.12.75) | 1-39 |
| X | US,A, 4132787 (REGNIER et al) 2 January 1979 (02.01.79) | 1-39 |
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| X | Patents Abstracts of Japan, C-249 page 9, JP,A, 59-116218 (KOUWA K.K.) 5 July 1984 (05.07.84) | 1-39 |
| X | Patents Abstracts of Japan, C-316, page 68, JP,A, 60-139621 (GRELAN SEIYAKU K.K.) 24 July 1985 (24.07.85) | 1-39 (continued) |

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"A" document defining the general state of the art which is not considered to be of particular relevance

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## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 1 October 1987 (01.10.87) | (09.10.87) 9 OCTOBER 1987 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| Australian Patent Office | J. BODEGRAVEN |

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FURTHER INFORMATI N CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
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| X | Derwent Soviet Inventions Illustrated, Vol.V, No.49, Issued 14 July 1975, Pharmaceuticals p.7, SU 406393 (TEVLINA) 10 July 1974 (10.07.74) | 1-39 |
| X | Derwent Abstract Accession No. 83-727103/31, class B04, SU,A, 959784 (MOSC MEDIAL STOMAT) 23 September 1982 (23.09.82) | 1-39 |
| X | McAuslan, B.R. and G.A. Gole (1980) Trans. Ophthalmol. Soc. U.K. 100 (3) 354-358 | 1-39 |

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-25 because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body by therapy. See Article 17(2)(b) and Rule 39.1(iv).

2. [ ] Claim numbers . . . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. [ ] Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00256

| Patent Document Cited in Search Report | Patent Family Members | | |
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END OF ANNEX